

RESEARCH ARTICLE

Pharmacological activation of the nuclear receptor REV-ERB reverses cognitive deficits and reduces amyloid- β burden in a mouse model of Alzheimer's disease

Deborah A. Roby¹, Fernanda Ruiz¹, Bailey A. Kermath¹, Jaymie R. Voorhees², Michael Niehoff³, Jinsong Zhang¹, John E. Morley³, Erik S. Musiek⁴, Susan A. Farr^{1,3*}, Thomas P. Burris^{2*}

1 Department of Pharmacology and Physiology, Saint Louis University School of Medicine, St. Louis, MO, United States of America, **2** Center for Clinical Pharmacology, Washington University School of Medicine and St. Louis College of Pharmacy, St. Louis, MO, United States of America, **3** Department of Internal Medicine, Saint Louis University School of Medicine, St. Louis, MO, United States of America, **4** Department of Neurology, Washington University School of Medicine, St. Louis, MO, United States of America

* burristhomas@wustl.edu (TPB); Susan.farr@health.slu.edu (SAF)



OPEN ACCESS

Citation: Roby DA, Ruiz F, Kermath BA, Voorhees JR, Niehoff M, Zhang J, et al. (2019) Pharmacological activation of the nuclear receptor REV-ERB reverses cognitive deficits and reduces amyloid- β burden in a mouse model of Alzheimer's disease. PLoS ONE 14(4): e0215004. <https://doi.org/10.1371/journal.pone.0215004>

Editor: Masuo Ohno, Nathan S Kline Institute, UNITED STATES

Received: September 14, 2018

Accepted: March 26, 2019

Published: April 11, 2019

Copyright: © 2019 Roby et al. This is an open access article distributed under the terms of the [Creative Commons Attribution License](https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Data Availability Statement: All relevant data are within the manuscript and its Supporting Information files.

Funding: This research was supported by grants from the National Institutes of Health to TPB (MH093429 and AG060769) and the U.S. Department of Veterans Affairs (to SAF). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Alzheimer's disease currently lacks treatment options that effectively reverse the biological/anatomical pathology and cognitive deficits associated with the disease. Loss of function of the nuclear receptor REV-ERB is associated with reduced cognitive function in mouse models. The effect of enhanced REV-ERB activity on cognitive function has not been examined. In this study, we tested the hypothesis that enhanced REV-ERB function may enhance cognitive function in a model of Alzheimer's disease. We utilized the REV-ERB agonist SR9009 to pharmacologically activate the activity of REV-ERB in the SAMP8 mouse model of Alzheimer's disease. SR9009 reversed cognitive dysfunction of an aged SAMP8 mouse in several behavioral assays including novel object recognition, T-maze foot shock avoidance, and lever press operant conditioning task assessments. SR9009 treatment reduced amyloid- β 1–40 and 1–42 levels in the cortex, which is consistent with improved cognitive function. Furthermore, SR9009 treatment led to increased hippocampal PSD-95, cortical synaptophysin expression and the number of synapses suggesting improvement in synaptic function. We conclude that REV-ERB is a potential target for treatment of Alzheimer's disease.

Introduction

REV-ERBs (REV-ERB α and β) are nuclear receptors that function as a ligand-dependent suppressors of gene transcription and are a critical components of the mammalian circadian clock [1–5]. Additionally, the REV-ERBs play important roles in physiological pathways including metabolism and inflammation [6–8]. Although their role in circadian behavior has been known for some time, more recent studies suggest that they also play an important role in learning and memory. *Rev-erba* null mice display impaired short-term, long-term, and

Competing interests: The authors have declared that no competing interests exist.

contextual memory and increased hippocampal neurogenesis [9–11]. REV-ERB recruits corepressors and class 1 histone deacetylases (HDACs) to target gene promoters and class 1 HDACs have also been implicated in learning and memory [12]. A number of synthetic agonists that activate REV-ERBs' transcriptional suppressor activity have been designed to assess the effects of modulating REV-ERB activity in vivo [13,14], but no studies examining their effects on cognitive function have been described.

Impaired cognitive function is a hallmark of Alzheimer's disease (AD) [15,16] and is characterized by accumulation of senile plaques composed of insoluble Amyloid- β ($A\beta$) peptides [17], neurofibrillary tangles [18], synaptic failure [19,20], neuroinflammation [21], and mitochondrial dysfunction [22]. Interestingly, disruption of normal sleep cycles and the circadian rhythm is also closely associated with AD [23], suggesting manipulation of these pathways may hold utility in treatment or prevention of the disease. A number of animal models have been developed for studying AD pathogenesis as well as in evaluation of novel therapies, which include very commonly utilized genetically modified mice that carry mutations in genes identified in patients with familial AD [24]. However, the majority of cases of AD are polygenic, or sporadic in nature [25]. The senescence accelerated mouse prone 8 (SAMP8) is a model of sporadic AD where the mice display age-related severe cognitive deficits by 12 months of age [26–28]. They develop pathological features of AD such as impaired $A\beta$ efflux from the brain [29,30], hyper-phosphorylated tau [31], elevated amyloid precursor protein [32,33], markers of synaptic dysfunction [34], and key changes in hippocampal gene expression [35].

Given that genetic REV-ERB α loss of function results in cognitive impairment [9], we sought to determine if pharmacological REV-ERB gain of function may improve cognitive function in a model of cognitive impairment and in particular AD. In the current study, we used the SAMP8 AD mouse model to evaluate whether a REV-ERB agonist (SR9009) could alter the time-dependent cognitive decline observed in these mice. We determined that SR9009 reverses cognitive deficits in the SAMP8 model in multiple cognitive assays by reducing $A\beta$ levels and rescuing glial and neuronal health.

Materials and methods

Animals and treatment

Experimentally naïve male SAMP8 mice (virus-free in-house colony) ages 4 months and 12 months were held in a temperature-controlled facility under 12–12 light and dark conditions (light on at 6 AM). Food and water were provided ad libitum. This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Animal care and experimental protocols used in this study were approved by the Saint Louis University Institutional Animal Care and Use Committee (Assurance number: 2532). SAMP8 animals were divided into 3 groups: 4-month-old mice receiving vehicle (Y-V), 12-month-old mice receiving vehicle (O-V), and 12-month-old mice receiving SR9009 (O-SR9009). The Rev-Erb agonist SR9009 was administered intraperitoneally once daily at Zeitgeber time (ZT) 0 at 100 mg/kg mouse body weight for 14 days before behavior testing commenced. Vehicle was 6% v/v DMSO and 10% v/v cremophor in water at a pH of 7. Total mice used was 72.

Novel object recognition

All behavioral assays for SAMP8 were conducted beginning two weeks post-onset of dosing. Learning and memory deficits were assayed using novel object recognition (NOR) as previously described [31]. SAMP8 mice were habituated to the maze on day 15, then exposed to two

identical objects on day 16 (acquisition). Twenty-four hours later on day 17, the animals were exposed to one familiar object and one novel object (retention). Time spent with novel object was calculated as a discrimination index. Group size was 11–17. Mice were tested between ZT2–ZT6. Time spent with novel object was calculated as a discrimination index (DI), defined as $(T_{\text{new}} - T_{\text{old}}) / (T_{\text{new}} + T_{\text{old}})$, as previously [36].

T-maze foot-shock avoidance

T-maze assay was conducted as previously described [37–39]. Briefly, animals were not permitted to explore the maze prior to testing. Animals were placed in the apparatus, and simultaneously a buzzer sounded while the start box door was raised. A constant, 0.35 mA foot shock began after 5 seconds. Mice avoided the shock by entering a clear escape box in one of the arms of the T-maze, and mice were trained to number of trials necessary to learn avoidance of the foot shock (acquisition). After one week, the number of trials required for the animals to make 5 avoidances out of 6 trials (retention) were recorded. Group size was 9–12. Mice were tested between ZT2–ZT6.

Lever press

Animals were habituated to evaporated milk with sucrose overnight for 3 days prior to testing. On the final night of habituation consumed volume was measured. Animals were separated into two groups and tested every other day for a total of 4 testing days. Animals were fasted overnight and placed in clear lever press boxes for 40 minutes per animal. Lever presses and reward retrievals were measured using the Coulbourn Instruments LabLinc system. Data was analyzed on Graphic State 3.02 software. Animals were trained for 1 day, wherein if the animals pressed the lever, the reward delivery arm was raised for 11 seconds. The subsequent 4 days, trial days, when the lever was pressed, the reward was available for 6 seconds. The number of each rewarded lever press was recorded (reward retrieval). Group size was 9–12. Mice were tested between ZT2–ZT6.

Amyloid- β analysis

Soluble A β was analyzed using ELISA. ELISA services provided by Confluence Discovery Technologies in St. Louis, MO. Kit was V-PLEX A β Peptide Panel 1 (4G8) for human, mouse, and rat (#Cat: K15199E-1) from Meso Scale Diagnostics, LLC. Cortex tissue was homogenized in 500 μ L reassembly buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, Pierce protease and phosphatase inhibitor mini tablets [Thermo Fisher], 20 mM NaF, 1 mM Na₃VO₄, 0.5 mM MgSO₄). Samples were incubated on ice for 30 min, then centrifuged at 150,000 g for 20 min at 4°C. Supernatants were then transferred to 50 μ L 0.5% Tween-20 in reassembly buffer, mixed and stored in 105 μ L aliquots as reassembly buffer-soluble fraction. Group size was 5.

Immunoblotting

Brain tissue was rapidly dissected out from hippocampus and temporal cortex and flash frozen in liquid nitrogen. Tissue samples were homogenized in RIPA buffer with protease and phosphatase inhibitors (Roche). Samples were centrifuged, and protein was collected from supernatant. Primary antibodies were PSD-95 (ThermoFisher, MA1-045) at 1:1000, synaptophysin (Abcam, ab32127) at 1:1000, beta-actin (mAbcam 8226) at 1:5000. Secondary antibodies were HRP-tagged anti-mouse and anti-rabbit (Santa Cruz) 1:5000. Group size was 3.

Histology

SAMP8 animals were anesthetized with 10 mg/mL ketamine HCl and 1 mg/mL xylazine at a dosage of 0.1 mL/10g mouse body weight injected i.p. The thoracic cavity was opened, and animals were perfused transcardially with 20 mL of phosphate buffered saline (PBS) and heparin. The brains were subsequently removed, halved, and the left hemisphere fixed in 4% paraformaldehyde, then in 30% sucrose to aid in sectioning. Brains were then serially sectioned at 50 μ m on a microtome with tris buffered saline (TBS) and sucrose and preserved in 30% ethylene glycol cryoprotectant. Sections were then processed free-floating in Netwells for GFAP (Dako) at 1:500 and ZO-1 (Thermo-Fisher) at 1:250 for immunofluorescence. Alexa Fluor 488 goat anti-rat and Alexa Fluor 568 goat anti-rabbit were used at 1:5000. 2 sections per mouse were analyzed. Co-staining quantification and measurement of blood vessel diameter were performed with ImageJ. Sections were viewed on either an Olympus BX41 EpiFluor or an Olympus FV1000 Confocal microscope. Group size was 5.

Transmission electron microscopy

Tissue was prepared as described [40]. Briefly, SAMP8 animals were anesthetized with 10 mg/mL ketamine HCl and 1 mg/mL xylazine at a dosage of 0.1 mL/10g mouse body weight. They were then perfused transcardially with PBS and 2.5% glutaraldehyde. Tissue sections were removed from the left hemisphere of the brain and pieces no more than 2mm³ were cut to contain both hippocampus and cortex. Tissue was then prepared according to protocol and viewed in a JEOL 1400 Plus electron microscope equipped with an AMT digital camera. 4 sections per group were analyzed for axon health. Sections were also imaged in cortex and total number of synapses were counted in each image. Group size was 4.

Statistical analysis

Statistical analysis for tests containing three test groups was conducted using 1-way ANOVA or 2-way ANOVA. We used Newman-Keuls multiple comparisons test to identify significantly different means.

Results and discussion

Since genetic *Rev-erba* loss of function results in reduced cognitive function, we hypothesized that increased REV-ERB activity may increase cognitive function in diseases where cognitive function is impaired. We used the SAMP8 mouse model of premature aging and AD to assess the ability of a REV-ERB agonist to alter cognitive function. We treated one set of “old” 12-month-old SAMP8 mice with SR9009 (O-SR9009; 14 days, 100 mg/kg q.d.), one set of 12-month-old SAMP8 mice with vehicle (O-V), and one set of “young” 4-month-old SAMP8 mice with vehicle (Y-V) and subjected them to three different behavioral tasks: novel object recognition (NOR), T-maze foot shock avoidance, and lever press operant conditioning. In the NOR test, reduced time spent exploring a novel object (reduced discrimination index) is associated with reduced cognitive function (reduction in the natural tendency of mice to investigate novel aspects of their environment). The hippocampus is involved in the 24-hour consolidation period between the introduction and recollection of novel objects [41], and this hippocampal-dependent memory assay is low stress and exposes cognitive impairments similar to those found in AD.

As expected, the O-V SAMP8 mice displayed decreased cognitive function in all the tests relative to the Y-V SAMP8 mice, and in nearly every case, SR9009 treatment resulted in significantly improved cognitive function in the O-SR9009 mice relative to the O-V mice (Fig 1). In

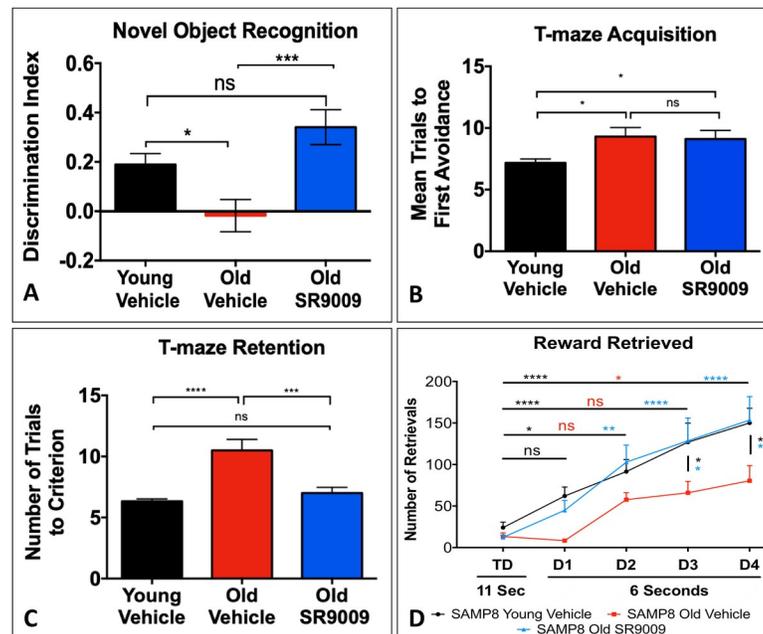


Fig 1. Reversal of cognitive deficits by SR9009. In novel object recognition, O-SR9009 mice had better recall of the old object and spent more time with the new (A). $DI = (T_{\text{new object}} - T_{\text{old object}}) / (T_{\text{new object}} + T_{\text{old object}})$ ($N = 11-17$). In T-maze, there was no significant difference between O-SR9009 and O-V during acquisition, but neither was there significant difference between Y-V and O-SR9009 (B) ($N = 9-12$). O-SR9009 mice had improved retention over O-V after one week (C). In the lever press task, O-SR9009 and Y-V mice retrieved more rewards over time than O-V and more rewards overall than O-V (D) ($N = 9-12$). These data together indicate rescued cognition in the SAMP8 mouse after up-regulation of Rev-Erb activity. * $p < 0.05$ *** $p < 0.001$ **** $p < 0.0001$.

<https://doi.org/10.1371/journal.pone.0215004.g001>

many cases, the cognitive function of O-SR9009 mice was indistinguishable from the young mice. In the NOR test, the discrimination index was clearly reduced in the O-V group relative to the Y-V group, consistent with reduced cognitive function with aging. Most importantly, the O-SR9009 group displayed a discrimination index that was significantly greater than the O-V group and was indistinguishable from the Y-V group (Fig 1A).

In the T-maze foot shock avoidance task, SR9009 treatment significantly improved retention in the mice, but the effect in acquisition was less pronounced. During the acquisition phase, the O-V group displayed a significant increase in the number of mean trials to first avoidance relative to Y-V mice, consistent with age-dependent reduction in cognitive function. O-SR9009 mice were not significantly improved over O-V mice, but neither did they have significantly more trials to first avoidance than Y-V mice (Fig 1B). However, one-week post-acquisition (retention), while the O-V mice still displayed reduced cognitive function, the SR9009 treated old mice displayed results indistinguishable from the Y-V mice (Fig 1C).

SR9009 treatment also resulted in improved performance in a lever press reward conditioning assay. Previous studies show that SAMP8 mice are impaired in the lever press operant conditioning task [26]. The O-V mice retrieved fewer rewards than young mice (Fig 1D). SR9009 treatment of the old mice resulted in reward retrieval that was indistinguishable from the young mice and significantly greater than the old mice treated with vehicle alone (Fig 1D). Taken together, these data indicate that SR9009 treatment improves cognitive function in a mouse model of AD. No alteration in the levels of Rev-erb α or Rev-erb β were noted in the brain with SR9009 treatment (S1 Fig). Using transmission electron microscopy, we also assessed the number of synapses in the hippocampal sections from each group of mice. We

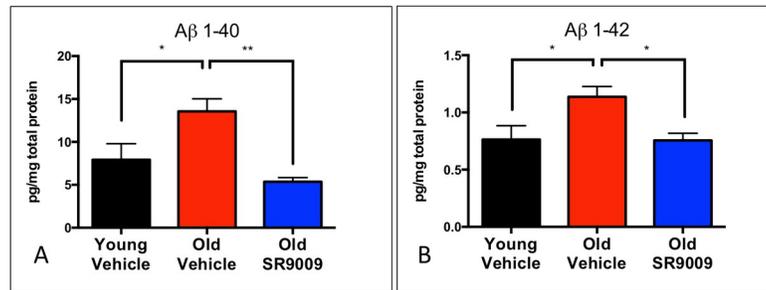


Fig 2. SR9009 lowers both Aβ 1–40 and Aβ 1–42 in the brain. Both species of Aβ were quantified by ELISA. Aβ 1–40 was significantly decreased (A). Aβ 1–42 was significantly decreased (B). *p<0.05 **p<0.01.

<https://doi.org/10.1371/journal.pone.0215004.g002>

observed a significant decreased in synapses in the aged SAMP8 mice that was reversed with SR9009 treatment.

Aβ peptides 1–40 and 1–42 are the most recognized pathological peptides in AD [42] due to their oligomerization leading to senile plaque formation that contributes to cognitive impairment [43]. Aβ 1–42 levels are positively correlated with the age of onset for AD [44]. Our lab and others have shown amyloid accumulation in the SAMP8 mouse [29,32,33] and consistent with previous studies, we observed significantly increased Aβ 1–40 and Aβ 1–42 levels in the brains of O-V mice (Fig 2). Most importantly, we found that treatment with SR9009 was associated with significant reduction in both species of Aβ peptides (Fig 2). The levels of both Aβ 1–40 and 1–42 were actually reduced to levels indistinguishable from those observed in the young SAMP8 mice (Fig 2).

Synaptic impairment contributes to loss of cognitive function in AD [19,45]. We measured PSD-95 protein levels in the hippocampus as a marker of dendritic spine density. There was a strong trend towards reduced expression of PSD-95 in the O-V mice relative to the Y-V mice (Fig 3A), consistent with the decreased cognitive function that we observed. SR9009 treated old mice displayed increased PSD-95 protein levels relative to their vehicle treated counterparts with a level indistinguishable from the young mice (Fig 3A). Because signaling to the cortex is impaired in AD, we next investigated synaptophysin protein expression, a marker for functional synapses, in the temporal cortex of the AD mice. Interestingly, there were no differences in synaptophysin expression between the old and young vehicle treated mice, but SR9009 treatment of the old mice there was a significant increase in synaptophysin (Fig 3B).

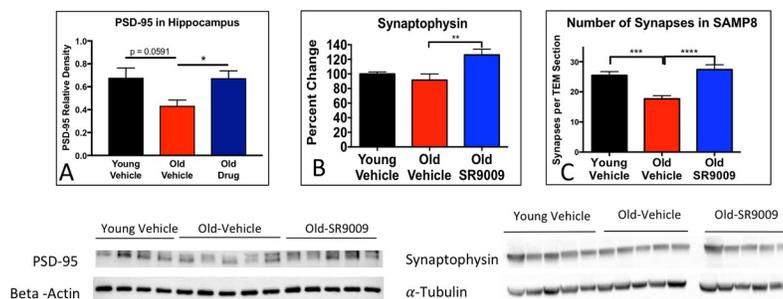


Fig 3. SR9009 treatment alters PSD-95 and synaptophysin expression and increases synapses. PSD-95 is a dendritic spine marker and its expression is down-regulated in the hippocampus of O-V mice. SR9009 treatment maintains levels of PSD-95 expression at levels of young mice (A). The synapse marker synaptophysin is increased by SR9009 treatment (B). The number of synapses identified per section in the cortex were quantified using TEM. TEM images were taken at 3000x magnification. (C). N = 5 * p<0.05.

<https://doi.org/10.1371/journal.pone.0215004.g003>

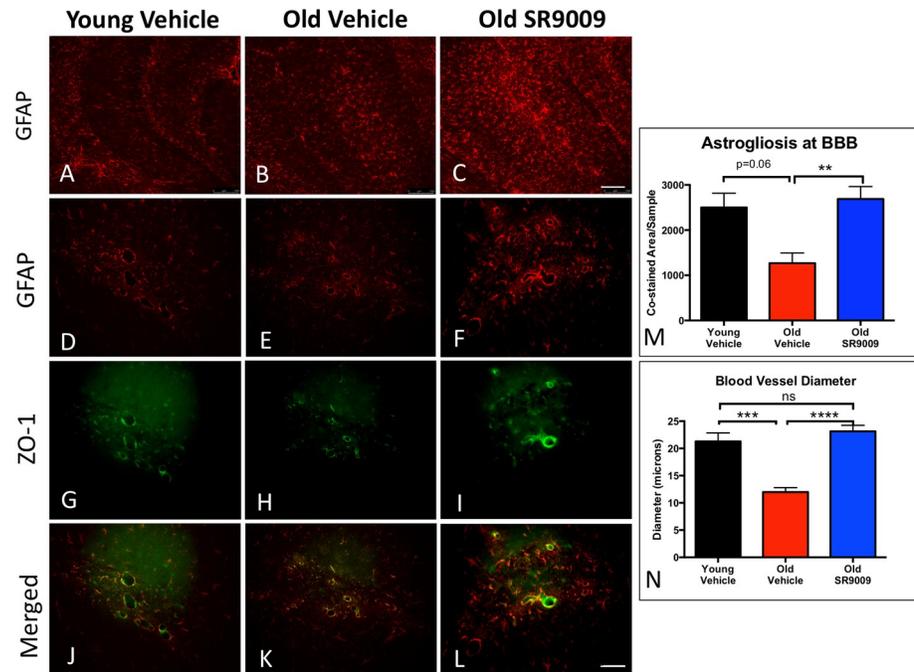


Fig 4. SR9009 increases astroglia in hippocampus, providing better support at the blood-brain barrier. IF staining for GFAP shows increase in activated astrocytes in dentate gyrus and CA3 neurons in the hippocampus of O-SR9009. There was very little GFAP staining in Y-V (A). GFAP was more pronounced in O-V (B) and even more pronounced in O-SR9009 (C). Co-staining GFAP (red) with ZO-1 (green), an endothelial marker, displayed GFAP and ZO-1 double staining in all mice (Y-V:D, G, J O-V:E, H, K O-SR9009: F, I, L). Consistent with the GFAP staining alone (A-C), there is more GFAP staining overall in O-SR9009 mice (F, I, L). The total co-stained area was greater in O-SR9009 than O-V, but not significant between Y-V and O-SR9009 (M). The diameter of blood vessels in the hippocampus were significantly greater in both Y-V and O-SR9009 than O-V, but there was no significant difference between Y-V and O-SR9009 (N). Scale bar in L consistent for D-L: 50 μ m. Scale bar for A-C in image, 100 μ m. * $p < 0.05$ *** $p < 0.001$ **** $p < 0.0001$.

<https://doi.org/10.1371/journal.pone.0215004.g004>

Conversely, we also assessed PSD-95 expression in the cortex and synaptophysin expression in the hippocampus. Age had no effect on expression of either of these proteins; however, SR9009 treatment did increase synaptophysin expression in the hippocampus (S2 Fig).

Blood brain barrier (BBB) dysfunction has been shown to be present in AD and has been suggested to play a role in the pathogenesis of this disease. In order to determine if we altered BBB functionality with SR9009 treatment, we examined the expression of the astrocyte marker, GFAP, by immunofluorescence in the hippocampus. We observed an increase in GFAP positive activated astrocytes in O-SR9009 mice relative to either the young and old vehicle treated mice (Fig 4A–4C).

Functional astrocytes maintain the integrity of the BBB, so we used ZO-1 as an endothelial marker for blood vessels and co-stained with GFAP for astrocytes. Consistent with our staining for GFAP alone, we found that there was more robust staining for GFAP overall in the O-SR9009 group than in the other groups (Fig 4D–4F). Co-staining for GFAP and ZO-1 displayed a strong trend towards reduction in O-V mice relative to Y-V mice and importantly, SR9009 treatment (O-SR9009) maintained co-staining levels that were equivalent to Y-V and significantly elevated compared to O-V mice (Fig 4M). Analysis of co-stained area reveals a trend towards reduced signal in the old vehicle treated mice (Fig 4M). SR9009 treatment of the old mice led to an increase in co-stained area relative to the old mice that were vehicle-treated (Fig 4M). Vessel diameter, which is characteristically reduced in AD [46,47], was also assessed.

Old vehicle treated mice displayed significantly reduced vessel diameter relative to the young mice and SR9009 treatment in the old mice substantially increased vessel size so there was no difference between Y-V and O-SR9009 (Fig 4N).

Axoskeletal abnormalities are also commonly observed in AD [48,49]. Using transmission electron microscopy, we readily observed abnormalities in oligodendrocyte myelination and neurofilaments in the old SAMP8 mouse. Axoskeletal aberrations arising in the old vehicle treated group that were not observed in the young or old SR9009 treated mice are shown in Fig 5. In the O-V mice, neurofilaments displayed loss of inter-axonal striatal structure, suggesting a disruption in axonal transport and a decrease in the number of healthy axons. We also observed significant deterioration of myelin structure in the old mice and interestingly, it was also noted in the young mice but to a lesser extent. O-SR9009 mice also displayed a deterioration of myelin structure that was similar to that of the Y-V mice.

Rev-erba null mice have demonstrated cognitive dysfunction characterized by deficits in working and short-term memory (Y-maze) and long-term memory (NOR and contextual fear conditioning) [9]. Other hippocampal dependent behaviors such as nest building were also deficient [10]. Therefore, loss of REV-ERB function leads to cognitive deficits. Consistent with the genetic loss of function of REV-ERB causing reduced cognitive function, in the current study, we found that pharmacological gain of function of REV-ERB was sufficient to reverse multiple cognitive deficits in the SAMP8 mouse model of sporadic AD. The small molecule REV-ERB agonist, SR9009, increases the transcriptional repressor activity of this nuclear receptor, allowing us to study gain-of-function. Aged SAMP8 mice treated with SR9009 performed better than aged SAMP8 mice treated with vehicle in NOR, T-maze foot shock avoidance, and lever press operant conditioning assessments. It is clear that activation of REV-ERB led to an improvement in the performance of hippocampal-dependent memory tasks.

The long term NOR assay (24 hours) is a hippocampus-dependent task, as is the T-maze foot shock avoidance assay [41]. The hippocampus is responsible for consolidating the recognition of the training object or avoidance of the foot shock into long-term memory. During acquisition for the T-maze task, while the SR9009 treated mice did not have significantly improved acquisition over the old vehicle treated mice, they did have improved retention of

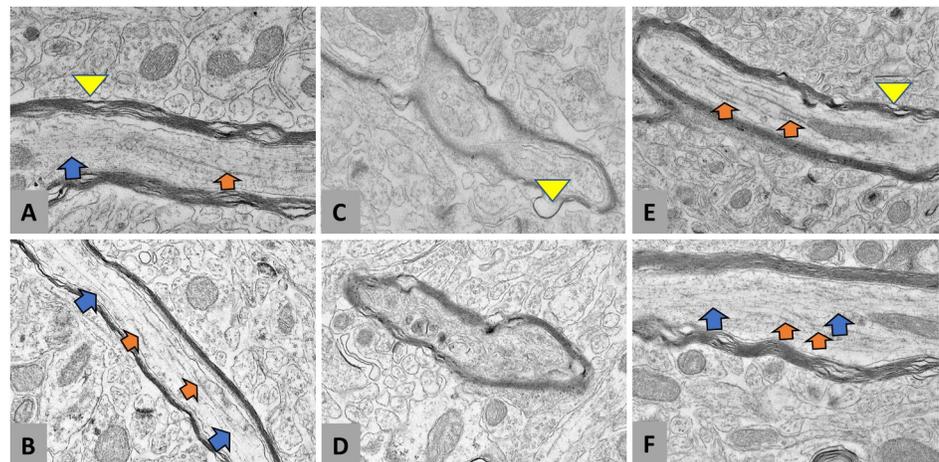


Fig 5. SR9009 rescues axoskeletal breakdown. Neurofilaments and microtubules supporting axonal structure in the Y-V group (A, B), O-V group (C, D), and O-SR9009 (E, F). Blue arrows depict neurofilament bundles and orange arrows depict microtubule sections. Both are missing in the axoskeletal structure of O-V mice. Aberrant myelination (yellow arrowheads) is found in all three groups, suggesting that the pathological breakdown in myelin structure begins before the onset of memory deficits.

<https://doi.org/10.1371/journal.pone.0215004.g005>

the task. This further suggests that hippocampal function is being rescued in the SAMP8 mouse model when treated with SR9009, as the hippocampus is still consolidating the task to long term memory.

Learning and memory also integrates multiple systems of memory, implicating the connectivity between hippocampus and striatal nuclei in cognitive tasks [50]. Here, we show that SR9009 improves the functional integration of multiple structures in the brain. While the mechanism is still unclear, we see in the old vehicle group that, even within the group, learning the task to retrieve a reward even compared to the previous test days does not reach a significant increase in reward retrieval until the final day of the test. This would suggest that, in the SAMP8 mouse, there is a breakdown in the integration of signals from the hippocampus and the limbic system, and that treatment with SR9009 reverses that breakdown.

Accumulation of A β peptides leads eventually to AD senile plaques. Prior to this pathological stage, amyloid precursor protein (APP) is processed at neuronal synapses, and A β oligomers are released into the extracellular matrix to have physiological effects [51]. A β in minimal amounts physiologically benefits memory, but excessive concentrations cause pathological effects [52]. We observed a clear reduction in A β 1–40 and 1–42 levels in mice treated with SR9009 with levels reaching those that were indistinguishable from the young mice. The mechanism underlying REV-ERB's reduction of A β levels is unclear, but the reduction is consistent with the improvement of cognitive function in the SR9009 treated mice. The decreased A β in the O-SR9009 group, in combination with improved cognition, suggests that increased A β levels are relevant in the SAMP8 model of AD and the levels are inversely correlated with cognitive function. Furthermore, A β toxicity is responsible for restructuring synapses in the brain [53,54], therefore decreased A β may be affecting synaptic plasticity in the SR9009-treated mice.

Diminishing synapses are one of the primary culprits in early loss of cognitive ability in the pathological progression of AD [55]. Synapse loss has long been documented in early and late forms of AD [45,56], and dendritic spine density correlates with improved learning and memory [57]. The increase in the pre- and post-synaptic markers PSD-95 and synaptophysin suggests that SR9009 alters cellular pathways leading to synaptic pruning in SAMP8 mice, thereby reversing the synaptic deficits in this AD model and leading to improved cognition.

Alongside the increase in synaptic proteins, we observe increased astrogliosis in the O-SR9009 group. This suggests that increased REV-ERB function improves cognition through positive glial activity. Astrocytes are known to be involved in synapse support as well as regulation of blood flow [58,59]. In the past, our lab has shown impaired blood brain barrier and A β efflux in SAMP8 mice [29,30,33]. Increased blood vessel diameter in the SR9009-treated mice suggests increased volume of blood flow throughout the brain. Multiple studies have shown that decrease in blood volume through the brain is common in AD and other dementias [60,61]. Pharmacological activation of REV-ERB appears to reverse the effects of decreased blood flow, both by increasing blood vessel diameter and through the increased astrocyte activity which supports blood vessels.

Typically, astrogliosis is a marker of neuronal injury. However, recent research suggests that upregulation of astrocytes is not always detrimental, but may be helpful in many circumstances, and the phenotype of reactive astrocytes will vary with type of injury [62–64]. As both Y-V and O-SR9009 animals retained good cognitive function, this suggests that the astrogliosis we observe in the old SR9009-treated SAMP8 mice is not detrimental to the animal. Functional astrocytes provide a support network for the high energy demand of the neural cells [59], thus it is possible that the astrogliosis that we observe is merely correlating to an increase in support for more active neurons in the SR9009 treated mice. This suggests that, as a negative regulator of gene expression, REV-ERB may be suppressing the toxic activity of reactive astrocytes,

allowing for the neurotrophic phenotype to play a more central role. Of course, the most important endpoint for assessing potential toxic activity of the reactive astrocytes is that the cognitive function of SR9009-treated animals, which illustrate that any negative impact of astrogliosis is outweighed by the positive impact.

Breakdown in neurofilament structure exemplified an axonal abnormality unique to the O-V group. Neurofibrillary tangles are a hallmark of AD caused by hyperphosphorylated tau. However, it has been suggested that breakdown in microtubule structure precedes the hyperphosphorylation of tau [65]. The disrupted structures that appear in the axons of O-V SAMP8 mice support this, as there was no change in hyperphosphorylation of tau between groups (S3 Fig). The O-SR9009 mice show no signs of the abnormal neurofibrillary structure, suggesting that gain of REV-ERB function reverses the downstream effect that causes the breakdown in axoskeletal structure. The axon carrying the signal must not only remain energetically efficient, but also maintain structural integrity in order for efficient neurotransmission. In the SAMP8, it appears that healthy axon structure contributes significantly to cognitive health. Breakdown in neurofilaments and microtubules in the axons of the 12-month-old mice damages cognition in the SAMP8. To our knowledge, this is the first work to show that the axons in a 12-month-old SAMP8 mouse form these tangle-like structures.

In human studies, patients with progressive dementia exhibit a loss of neurofilament heavy and light chain proteins as compared to their age-matched non-demented counterparts [66], which is similar to what is observed in the SAMP8 mouse model. Thus, the protective effect of SR9009 on degeneration of axonal structure in the SAMP8 mice may be relevant in human neurodegeneration.

Aberrant myelination has also been well-documented in AD [67,68]. In our study, we found that myelination erosion began early in life in the SAMP8 mouse but had not progressed sufficiently to cause cognitive impairment at 4 months of age. However, the breakdown in myelination was particularly apparent at 12 months of age. It is as yet unclear if this is due to a decrease in oligodendrocytes or a deficiency in the myelination process. However, by 12 months of age, the neuronal signaling was impaired to the point of cognitive impairment, but SR9009 was able to rescue the effects of myelination breakdown in the old mice. However, this is consistent with previous studies that show that, in neurodegenerative diseases such as AD, the deleterious effects on the brain begin immediately after adolescence [69]. Therefore, it is not surprising that the 4-month-old SAMP8 mice do not yet display the cognitive deficits of the 12-month-old control group while still displaying the abnormal myelination.

The endogenous ligand for both REV-ERB α and REV-ERB β is heme [70,71]. Although the physiological relevance of heme as an endogenous ligand is unclear, understanding of ligand regulation of REV-ERB has allowed development of synthetic ligands that have been important for understanding the relevance of the REV-ERBs as potential drug targets. The REV-ERBs are widely expressed, and heme is produced in all cells as a critical component (prosthetic group) of enzymes of cellular energetics, among other key roles. Previously, we demonstrated a role for variations in intracellular heme levels with the physiological role of REV-ERBs in adipogenesis [72]; however, the role of the endogenous REV-ERB ligand in terms of neuronal function and cognition is not clear. What is clear is that heme metabolism appears to be highly dysregulated in AD. Heme oxygenase 1 (HO-1), the key heme degrading enzyme induced by both heme and oxidative stress, is substantially upregulated in the brains of patients with AD. This is consistent with the supposition that AD pathology is associated with significant oxidative stress [73–75]. The typical physiological response to decreased heme levels is to increase the expression of the rate-limiting heme synthesis enzyme aminolevulinic synthase 1 (ALAS1), but in the case of AD the level of expression of ALAS1 was decreased by ~90% in the brains of patients [74]. Of course, increased expression of HO-1 (heme

degradation) and decreased expression of ALAS1 (heme synthesis) would lead to a situation where dysregulated heme levels could cause significant cellular stress and, from the perspective of REV-ERB, normal physiological activity would be decreased. Loss of REV-ERB α function is known to be associated with reduced cognitive function [9–11] and lower levels of heme would translate into less active REV-ERB α and REV-ERB β , thus reducing cognitive function. Currently, we do not know if loss of REV-ERB function leads to AD-like pathology beyond cognitive dysfunction. SR9009 treatment may activate REV-ERB to levels that are more aligned with physiological function if heme levels were normal, which may have a beneficial effect on neuronal function.

Conclusions

We have demonstrated that gain of REV-ERB function through pharmacological activation with SR9009 reversed cognitive defects in an AD mouse model. Mechanistically, we noted that SR9009 led to decreased A β levels in the brain as well as increased expression of markers of synaptic health and improved axoskeletal structure. These data clearly suggest that targeting of REV-ERB may be a method to effectively treat AD as well as other cognitive disorders.

Supporting information

S1 Fig. Rev-erb expression is not altered by SR9009 treatment. Cortex was rapidly dissected and flash frozen in liquid nitrogen for gene expression analysis. RNA was extracted using TRIzol extraction techniques, and gene expression of *Rev-erba* and β was analyzed using Real Time qPCR. There were no changes in REV-ERB- α or REV-ERB- β gene expression either in the old SAMP8 mice or those treated with SR9009.

(TIF)

S2 Fig. Expression of PSD-95 (Cortex) and synaptophysin (Hippocampus). PSD-95 and synaptophysin expression were assessed by western blot. Young (4-month old) or Old (12-month old) SAMP8 mice treated with vehicle were compared to old SAMP8 mice treated with SR9009 for 4 weeks.

(TIF)

S3 Fig. p-Tau T231 levels are not altered by SR9009 treatment. Western blot assessment of p-Tau T231 revealed no differences between Y-V, O-V and O-SR9009 treated groups.

(TIF)

Acknowledgments

The authors would like to thank Dr. Grant Kolar, Barbara Nagel, and Caroline Murphy for their assistance with histology.

Author Contributions

Conceptualization: Deborah A. Roby, Susan A. Farr, Thomas P. Burris.

Formal analysis: Deborah A. Roby, Jaymie R. Voorhees, Michael Niehoff, Jinsong Zhang, Erik S. Musiek.

Funding acquisition: John E. Morley, Susan A. Farr, Thomas P. Burris.

Investigation: Deborah A. Roby, Fernanda Ruiz, Bailey A. Kermath, Jaymie R. Voorhees, Michael Niehoff.

Methodology: Susan A. Farr.

Project administration: Susan A. Farr, Thomas P. Burris.

Supervision: Susan A. Farr, Thomas P. Burris.

Visualization: Deborah A. Roby.

Writing – original draft: Deborah A. Roby.

Writing – review & editing: Erik S. Musiek, Susan A. Farr, Thomas P. Burris.

References

1. Burris TP. Nuclear hormone receptors for heme: REV-ERB α and REV-ERB β are ligand-regulated components of the mammalian clock. *Mol Endocrinol*. 2008/01/24. 2008; 22(7):1509–20. <https://doi.org/10.1210/me.2007-0519> PMID: 18218725
2. Crumbley C, Wang Y, Kojetin DJ, Burris TP. Characterization of the core mammalian clock component, NPAS2, as a REV-ERB α /ROF α target gene. *J Biol Chem*. 2010; 285(46):35386–92. <https://doi.org/10.1074/jbc.M110.129288> PMID: 20817722
3. Crumbley C, Burris TP. Direct regulation of CLOCK expression by REV-ERB. *PLoS One*. 2011; 6(3): e17290. <https://doi.org/10.1371/journal.pone.0017290> PMID: 21479263
4. Preitner N, Damiola F, Lopez-Molina L, Zakany J, Duboule D, Albrecht U, et al. The orphan nuclear receptor REV-ERB α controls circadian transcription within the positive limb of the mammalian circadian oscillator. *Cell*. 2002; 110(2):251–60. PMID: 12150932
5. Cho H, Zhao X, Hatori M, Yu RT, Barish GD, Lam MT, et al. Regulation of circadian behaviour and metabolism by REV-ERB α and REV-ERB β . *Nature*. 2012/03/29. 2012; 485(7396):123–7. <https://doi.org/10.1038/nature11048> PMID: 22460952
6. Duez H, Staels B. Rev-erb-a: an integrator of circadian rhythms and metabolism. *J Appl Physiol*. 2009/08/20. 2009; 107(6):1972–80. <https://doi.org/10.1152/jappphysiol.00570.2009> PMID: 19696364
7. Duez H, Staels B. Rev-erba gives a time cue to metabolism. *FEBS Lett*. 2007/08/24. 2008; 582(1):19–25. <https://doi.org/10.1016/j.febslet.2007.08.032> PMID: 17765229
8. Ramakrishnan SN, Muscat GE. The orphan Rev-erb nuclear receptors: a link between metabolism, circadian rhythm and inflammation? *Nucl Recept Signal*. 2006/04/28. 2006; 4:e009. <https://doi.org/10.1621/nrs.04009> PMID: 16741567
9. Schnell A, Chappuis S, Schmutz I, Brai E, Ripperger JA, Schaad O, et al. The nuclear receptor REV-ERB α regulates Fabp7 and modulates adult hippocampal neurogenesis. *PLoS One*. 2014; 9(6): e99883. <https://doi.org/10.1371/journal.pone.0099883> PMID: 24932636
10. Jager J, O'Brien WT, Manlove J, Krizman EN, Fang B, Gerhart-Hines Z, et al. Behavioral changes and dopaminergic dysregulation in mice lacking the nuclear receptor Rev-erb α . *Mol Endocrinol*. 2014; 28(4):490–8. <https://doi.org/10.1210/me.2013-1351> PMID: 24552589
11. Valnegri P, Khelifaoui M, Dorseuil O, Bassani S, Lagneaux C, Gianfelice A, et al. A circadian clock in hippocampus is regulated by interaction between oligophrenin-1 and Rev-erba. *Nat Neurosci*. 2011/08/28. 2011; 14(10):1293–301. <https://doi.org/10.1038/nn.2911> PMID: 21874017
12. Schmauss C. The roles of class I histone deacetylases (HDACs) in memory, learning, and executive cognitive functions: A review. *Neurosci Biobehav Rev*. 2017; 83:63–71. <https://doi.org/10.1016/j.neubiorev.2017.10.004> PMID: 29017914
13. Solt LA, Wang Y, Banerjee S, Hughes T, Kojetin DJ, Lundasen T, et al. Regulation of circadian behaviour and metabolism by synthetic REV-ERB agonists. *Nature*. 2012; 485(7396):62–8. <https://doi.org/10.1038/nature11030> PMID: 22460951
14. Banerjee S, Wang Y, Solt LA, Griffett K, Kazantzis M, Amador A, et al. Pharmacological targeting of the mammalian clock regulates sleep architecture and emotional behaviour. *Nat Commun*. 2014; 5:5759. <https://doi.org/10.1038/ncomms6759> PMID: 25536025
15. Bishop NA, Lu T, Yankner BA. Neural mechanisms of ageing and cognitive decline. *Nature*. 2010; 464(7288):529–35. <https://doi.org/10.1038/nature08983> PMID: 20336135
16. Caterini F, Sala S Della, Spinnler H, Stangalino C, Turnbull OH. Object recognition and object orientation in Alzheimer's disease. *Neuropsychology*. 2002; 16(2):146–55. PMID: 11949706
17. Sadigh-Eteghad S, Sabermarouf B, Majdi A, Talebi M, Farhoudi M, Mahmoudi J. Amyloid-beta: a crucial factor in Alzheimer's disease. *Med Princ Pr*. 2014/11/27. 2015; 24(1):1–10.

18. Brion JP. Immunological demonstration of tau protein in neurofibrillary tangles of Alzheimer's disease. *J Alzheimers Dis.* 2006; 9(3 Suppl):177–85.
19. DeKosky ST, Scheff SW. Synapse loss in frontal cortex biopsies in Alzheimer's disease: correlation with cognitive severity. *Ann Neurol.* 1990; 27(5):457–64. <https://doi.org/10.1002/ana.410270502> PMID: 2360787
20. Spiers-Jones TL, Hyman BT. The intersection of amyloid beta and tau at synapses in Alzheimer's disease. *Neuron.* 2014; 82(4):756–71. <https://doi.org/10.1016/j.neuron.2014.05.004> PMID: 24853936
21. Pimplikar SW. Neuroinflammation in Alzheimer's disease: from pathogenesis to a therapeutic target. *J Clin Immunol.* 2014/04/08. 2014; 34 Suppl 1:S64–9.
22. Silva DF, Selfridge JE, Lu J, E L, Cardoso SM, Swerdlow RH. Mitochondrial abnormalities in Alzheimer's disease: possible targets for therapeutic intervention. *Adv Pharmacol.* 2012; 64:83–126. <https://doi.org/10.1016/B978-0-12-394816-8.00003-9> PMID: 22840745
23. Musiek ES, Xiong DD, Holtzman DM. Sleep, circadian rhythms, and the pathogenesis of Alzheimer Disease. 2015;
24. Esquerda-Canals G, Montoliu-Gaya L, Güell-Bosch J, Villegas S. Mouse Models of Alzheimer's Disease. *J Alzheimers Dis.* 2017; 57(4):1171–83. <https://doi.org/10.3233/JAD-170045> PMID: 28304309
25. Sun Q, Xie N, Tang B, Li R, Shen Y. Alzheimer's Disease: From Genetic Variants to the Distinct Pathological Mechanisms. *Front Mol Neurosci.* 2017/10/06. 2017; 10:319. <https://doi.org/10.3389/fnmol.2017.00319> PMID: 29056900
26. Miyamoto M. Characteristics of age-related behavioral changes in senescence-accelerated mouse SAMP8 and SAMP10. *Exp Gerontol.* 1997; 32(1–2):139–48. PMID: 9088911
27. Flood JF, Morley JE. Early onset of age-related impairment of aversive and appetitive learning in the SAM-P/8 mouse. *J Gerontology.* 1992; 47(2):52–9.
28. Flood JF, Morley JE. Learning and memory in the SAMP8 mouse. *Neurosci Biobehav Rev.* 1998; 22:1–20. PMID: 9491937
29. del Valle J, Duran-Vilaregut J, Manich G, Pallàs M, Camins A, Vilaplana J, et al. Cerebral amyloid angiopathy, blood-brain barrier disruption and amyloid accumulation in SAMP8 mice. *Neurodegener Dis.* 2011/03/16. 2011; 8(6):421–9. <https://doi.org/10.1159/000324757> PMID: 21411981
30. Erickson MA, Niehoff ML, Farr SA, Morley JE, Dillman LA, Lynch KM, et al. Peripheral administration of antisense oligonucleotides targeting the amyloid-beta protein precursor reverses AbetaPP and LRP-1 overexpression in the aged SAMP8 mouse brain. *J Alzheimers Dis.* 2012; 28(4):951–60. <https://doi.org/10.3233/JAD-2011-111517> PMID: 22179572
31. Farr SA, Ripley JL, Sultana R, Zhang Z, Niehoff ML, Platt TL, et al. Antisense oligonucleotide against GSK-3beta in brain of SAMP8 mice improves learning and memory and decreases oxidative stress: Involvement of transcription factor Nrf2 and implications for Alzheimer disease. *Free Radic Biol Med.* 2014; 67:387–95. <https://doi.org/10.1016/j.freeradbiomed.2013.11.014> PMID: 24355211
32. Morley JE, Kumar VB, Bernardo AE, Farr SA, Uezu K, Tumosa N, et al. Beta-amyloid precursor polypeptide in SAMP8 mice affects learning and memory. *Peptides.* 2000; 21(12):1761–7. PMID: 11150635
33. Del Valle J, Duran-Vilaregut J, Manich G, Casadesús G, Smith MA, Camins A, et al. Early amyloid accumulation in the hippocampus of SAMP8 mice. *J Alzheimers Dis.* 2010; 19(4):1303–15. <https://doi.org/10.3233/JAD-2010-1321> PMID: 20061609
34. Tomobe K, Okuma Y, Nomura Y. Impairment of CREB phosphorylation in the hippocampal CA1 region of the senescence-accelerated mouse (SAM) P8. *Brain Res.* 2007/02/14. 2007; 1141:214–7. <https://doi.org/10.1016/j.brainres.2006.08.026> PMID: 17303091
35. Armbrrecht HJ, Siddiqui AM, Green M, Farr SA, Kumar VB, Banks WA, et al. SAMP8 mice have altered hippocampal gene expression in long term potentiation, phosphatidylinositol signaling, and endocytosis pathways. *Neurobiol Aging.* 2014; 35(1):159–68. <https://doi.org/10.1016/j.neurobiolaging.2013.07.018> PMID: 23969180
36. Pedrós I, Petrov D, Allgaier M, Sureda F, Barroso E, Beas-Zarate C, et al. Early alterations in energy metabolism in the hippocampus of APP^{swe}/PS1^{dE9} mouse model of Alzheimer's disease. *Biochim Biophys Acta.* 2014/06/02. 2014; 1842(9):1556–66. <https://doi.org/10.1016/j.bbadis.2014.05.025> PMID: 24887203
37. Armbrrecht HJ, Siddiqui AM, Green M, Farr SA, Kumar VB, Banks WA, et al. Antisense against Amyloid-beta Protein Precursor Reverses Memory Deficits and Alters Gene Expression in Neurotrophic and Insulin-Signaling Pathways in SAMP8 Mice. *J Alzheimers Dis.* 2015; 46(2):535–48. <https://doi.org/10.3233/JAD-142760> PMID: 25854933
38. Farr SA, Banks WA, La Scola ME, Flood JF, Morley JE. Permanent and temporary inactivation of the hippocampus impairs T-maze footshock avoidance acquisition and retention. *Brain Res.* 2000; 872:242–9. PMID: 10924702

39. Morley JE, Farr SA, Flood JF. Antibody to amyloid beta protein alleviates impaired acquisition, retention, and memory processing in SAMP8 mice. *Neurobiol Learn Mem.* 2002; 78(1):125–38. <https://doi.org/10.1006/nlme.2001.4047> PMID: 12071671
40. Bauskar A, Mack WJ, Mauris J, Argüeso P, Heur M, Nagel BA, et al. Clusterin Seals the Ocular Surface Barrier in Mouse Dry Eye. *PLoS One.* 2015/09/24. 2015; 10(9):e0138958. <https://doi.org/10.1371/journal.pone.0138958> PMID: 26402857
41. Hammond RS, Tull LE, Stackman RW. On the delay-dependent involvement of the hippocampus in object recognition memory. *Neurobiol Learn Mem.* 2004; 82(1):26–34. <https://doi.org/10.1016/j.nlm.2004.03.005> PMID: 15183168
42. Murphy MP, Iii HL. Alzheimer's Disease and the β -Amyloid Peptide. *J Alzheimer's Dis.* 2010; 19(1):1–17.
43. Bitan G, Kirkitadze MD, Lomakin A, Vollers SS, Benedek GB, Teplow DB. Amyloid β -protein (A) assembly: A 40 and A 42 oligomerize through distinct pathways. *Proc Natl Acad Sci.* 2003; 100(1):330–5. <https://doi.org/10.1073/pnas.222681699> PMID: 12506200
44. Duering M, Grimm MOW, Grimm HS, Schröder J, Hartmann T. Mean age of onset in familial Alzheimer's disease is determined by amyloid beta 42. *Neurobiol Aging.* 2005; 26(6):785–8. <https://doi.org/10.1016/j.neurobiolaging.2004.08.002> PMID: 15718035
45. Terry RD, Masliah E, Salmon DP, Butters N, DeTeresa R, Hill R, et al. Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. *Ann Neurol.* 1991; 30(4):572–80. <https://doi.org/10.1002/ana.410300410> PMID: 1789684
46. MacVicar BA, Newman EA. Astrocyte regulation of blood flow in the brain. *Cold Spring Harb Perspect Biol.* 2015/03/27. 2015; 7(5).
47. de la Torre JC. Is Alzheimer's disease a neurodegenerative or a vascular disorder? Data, dogma, and dialectics. *Lancet Neurol.* 2004; 3(3):184–90. [https://doi.org/10.1016/S1474-4422\(04\)00683-0](https://doi.org/10.1016/S1474-4422(04)00683-0) PMID: 14980533
48. Braak E, Braak H, Mandelkow EM. A sequence of cytoskeleton changes related to the formation of neurofibrillary tangles and neuropil threads. *Acta Neuropathol.* 1994; 87(6):554–67. PMID: 7522386
49. Terwel D, Dewachter I, Van Leuven F. Axonal transport, tau protein, and neurodegeneration in Alzheimer's disease. *Neuromolecular Med.* 2002; 2(2):151–65. <https://doi.org/10.1385/NMM:2:2:151> PMID: 12428809
50. Johnson A, van der Meer MA, Redish AD. Integrating hippocampus and striatum in decision-making. *Curr Opin Neurobiol.* 2008/03/04. 2007; 17(6):692–7. <https://doi.org/10.1016/j.conb.2008.01.003> PMID: 18313289
51. O'Brien RJ, Wong PC. Amyloid precursor protein processing and Alzheimer's disease. *Annu Rev Neurosci.* 2011; 34:185–204. <https://doi.org/10.1146/annurev-neuro-061010-113613> PMID: 21456963
52. Morley JE, Farr SA, Banks WA, Johnson SN, Yamada KA, Xu L. A Physiological Role for Amyloid- β Protein: Enhancement of Learning and Memory. *J Alzheimer's Dis.* 2010; 19(2):441–9.
53. Mucke L, Selkoe DJ. Neurotoxicity of amyloid β -protein: synaptic and network dysfunction. *Cold Spring Harb Perspect Med.* 2012; 2:a006338. <https://doi.org/10.1101/cshperspect.a006338> PMID: 22762015
54. Parihar MS, Brewer GJ. Amyloid Beta as a Modulator of Synaptic Plasticity. Vol. 22, *Journal of Alzheimer's Disease.* 2011. 741–763 p.
55. Selkoe DJ. Alzheimer's disease is a synaptic failure. *Science (80-).* 2002; 298:789–91.
56. Davies C, Mann D, Sumpter P, Yates P. A quantitative morphometric analysis of the neuronal and synaptic content of the frontal and temporal cortex in patients with Alzheimer's disease. *J Neurol Sci.* 1987; 78(2):151–64. PMID: 3572454
57. Mahmoud RR, Sase S, Aher YD, Sase A, Gröger M, Mokhtar M, et al. Spatial and Working Memory Is Linked to Spine Density and Mushroom Spines. *PLoS One.* 2015/10/15. 2015; 10(10):e0139739. <https://doi.org/10.1371/journal.pone.0139739> PMID: 26469788
58. Attwell D, Buchan AM, Charkpak S, Lauritzen M, Macvicar BA, Newman EA. Glial and neuronal control of brain blood flow. *Nature.* 2010; 468(7321):232–43. <https://doi.org/10.1038/nature09613> PMID: 21068832
59. Halassa MM, Haydon PG. Integrated brain circuits: astrocytic networks modulate neuronal activity and behavior. *Annu Rev Physiol.* 2010; 72:335–55. <https://doi.org/10.1146/annurev-physiol-021909-135843> PMID: 20148679
60. Roher AE, Debbins JP, Malek-Ahmadi M, Chen K, Pipe JG, Maze S, et al. Cerebral blood flow in Alzheimer's disease. *Vasc Heal Risk Manag.* 2012/10/23. 2012; 8:599–611.

61. de Heus RAA, de Jong DLK, Sanders ML, van Spijker GJ, Oudegeest-Sander MH, Hopman MT, et al. Dynamic Regulation of Cerebral Blood Flow in Patients With Alzheimer Disease. *Hypertension*. 2018/05/29. 2018;
62. Liddelow SA, Guttenplan KA, Clarke LE, Bennett FC, Bohlen CJ, Schirmer L, et al. Neurotoxic reactive astrocytes are induced by activated microglia. *Nature*. 2017/01/18. 2017; 541(7638):481–7. <https://doi.org/10.1038/nature21029> PMID: 28099414
63. Zamanian JL, Xu L, Foo LC, Nouri N, Zhou L, Giffard RG, et al. Genomic analysis of reactive astrogliosis. *J Neurosci*. 2012; 32(18):6391–410. <https://doi.org/10.1523/JNEUROSCI.6221-11.2012> PMID: 22553043
64. Anderson M, Ao Y, Sofroniew M. Heterogeneity of reactive astrocytes. *Neurosci Lett*. 2014; 565:23–9. <https://doi.org/10.1016/j.neulet.2013.12.030> PMID: 24361547
65. Miyasaka T, Sato S, Tatebayashi Y, Takashima A. Microtubule destruction induces tau liberation and its subsequent phosphorylation. *FEBS Lett*. 2010; 584(14):3227–32. <https://doi.org/10.1016/j.febslet.2010.06.014> PMID: 20561519
66. Kester MI, Scheffer PG, Koel-Simmelink MJ, Twaalfhoven H, Verwey NA, Veerhuis R, et al. Serial CSF sampling in Alzheimer's disease: specific versus non-specific markers. *Neurobiol Aging*. 2011/07/07. 2012; 33(8):1591–8. <https://doi.org/10.1016/j.neurobiolaging.2011.05.013> PMID: 21741127
67. Migliaccio R, Agosta F, Possin KL, Rabinovici GD, Miller BL, Gorno-Tempini ML. White matter atrophy in Alzheimer's disease variants. *Alzheimers Dement*. 2012; 8(5 Suppl):S78–87.e1-2.
68. Teipel SJ, Bayer W, Alexander GE, Zebuhr Y, Teichberg D, Kulic L, et al. Progression of corpus callosum atrophy in Alzheimer disease. *Arch Neurol*. 2002; 59(2):243–8. PMID: 11843695
69. Salthouse TA. When does age-related cognitive decline begin? *Neurobiol Aging*. 2009/02/20. 2009; 30(4):507–14. <https://doi.org/10.1016/j.neurobiolaging.2008.09.023> PMID: 19231028
70. Raghuram S, Stayrook KR, Huang P, Rogers PM, Nosie AK, McClure DB, et al. Identification of heme as the ligand for the orphan nuclear receptors REV-ERB α and REV-ERB β . *Nat Struct Mol Biol*. 2007; 14(12):1207–13. <https://doi.org/10.1038/nsmb1344> PMID: 18037887
71. Yin L, Wu N, Curtin JC, Qatanani M, Szwegold NR, Reid RA, et al. Rev-erba, a heme sensor that coordinates metabolic and circadian pathways. *Science* (80-). 2007/11/15. 2007; 318(5857):1786–9.
72. Kumar N, Solt LA, Wang Y, Rogers PM, Bhattacharyya G, Kamenecka TM, et al. Regulation of adipogenesis by natural and synthetic REV-ERB ligands. *Endocrinology*. 2010; 151(7):3015–25. <https://doi.org/10.1210/en.2009-0800> PMID: 20427485
73. Smith M a Kutty RK, Richey PL Yan SD, Stern D Chader GJ, et al. Heme oxygenase-1 is associated with the neurofibrillary pathology of Alzheimer's disease. *Am J Pathol*. 1994; 145(1):42–7. PMID: 8030754
74. Premkumar DR, Smith MA, Richey PL, Petersen RB, et al. Induction of Heme Oxygenase-1 mRNA and Protein in Neocortex and Cerebral Vessels in Alzheimer's Disease. *J Neurochem*. 1995; 65(3):1399–402. PMID: 7543935
75. Dwyer BE, Smith MA, Richardson SL, Perry G, Zhu X. Down-regulation of aminolevulinic acid synthase, the rate-limiting enzyme for heme biosynthesis in Alzheimer's disease. *Neurosci Lett*. 2009; 460(2):180–4. <https://doi.org/10.1016/j.neulet.2009.05.058> PMID: 19477221